Orderly wheels of the cyanobacterial clock

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Circadian rhythms, self-sustaining oscillations in an organism's behavior that repeat roughly once per day, have fascinated biologists for hundreds of years (1, 2). Part of this fascination comes from the fact that the existence of intrinsic biological rhythms implies that an organism carries within it a model of how it expects the external world to behave—a plant that moves its leaves in anticipation of the sun even when grown in constant conditions must have some hard-wired knowledge of the rotation of the Earth. Fundamentally, these predictive biological rhythms must originate mechanistically from carefully regulated molecular interactions that generate oscillations. In PNAS, Chang et al. (3) present a series of cleverly designed structural and biochemical experiments that shed light on the inner workings of the best-defined biological clock, the KaiABC protein oscillator.

Although it may not be surprising that complex multicellular organisms like fruit flies and humans are capable of creating predictive models of their external environments, autonomous circadian rhythms can frequently be found in free-living single cells as well (4). The simplest genetic model organism we have today that exhibits circadian rhythms is the cyanobacterium *Synechococcus elongatus*, a photosynthetic microbe with a clock that controls pronounced oscillations in gene expression (5). The heart of this cyanobacterial clock is formed by the interaction of three proteins, KaiA, KaiB, and KaiC. Following methods pioneered by Takao Kondo and coworkers (6), a ∼24-h rhythm in KaiC phosphorylation can be reconstituted using only these purified proteins (Fig. 1 *A* and *B*).

This remarkable finding essentially reduced the puzzle of how a biological rhythm is generated to the problem of understanding the chemistry of three known components. KaiC is an unusual relative of the AAA+ superfamily of ATPases with very slow enzymatic activity (7, 8). The protein forms hexameric rings in the presence of ATP in which each KaiC monomer consists of two homologous catalytic domains, CI and CII (9). The KaiC hexamer thus appears as a "double doughnut," with the CII ring atop the CI ring. The CI domain is an ATPase, but the CII domain has acquired additional phosphotransferase activities: it can phosphorylate and dephosphorylate itself at CII residues near the monomer–monomer interfaces, notably Ser431 and Thr432, and does so in a specific ordered pattern throughout the course of a day (10–12). At times corresponding to the subjective morning, KaiA interacts with a sequence at the C terminus of CII and promotes kinase activity, leading to phosphorylation (13, 14). KaiB, a protein with a thioredoxin-like fold, is able to interact with appropriately phosphorylated KaiC near subjective dusk, and these KaiB·KaiC complexes inhibit KaiA, allowing dephosphorylation to occur and the cycle to repeat (11, 12).

This verbal description of the events in the circadian cycle does not itself give a satisfactory account of what the period of the clock should be, how the system depends on the concentration of the components, or, indeed, whether stable oscillations should occur at all. Unlike the familiar oscillations of a swinging bob in a pendulum clock, oscillating chemical reactions are fundamentally nonlinear. That is, a cycle of elementary reactions, one leading to the next, that ultimately brings the system back to its starting state, can never itself produce stable oscillations. To drive oscillations, there must also be feedback interactions in the reaction network, strong enough and appropriately arranged to enforce synchrony and push the system away from steady state.

A significant amount of mathematical modeling work has been done on the KaiABC system in an attempt to define the critical feedback interactions, often identifying the phosphorylation-dependent sequestration of KaiA into protein–protein complexes as a crucial event (12, 15, 16). These models have identified core principles of how the clock functions, but they leave many of the conspicuous biochemical features of the Kai proteins unexplained. For example, KaiC is not a traditional protein kinase, but instead is an ATPase that has expanded its "skill set" to include autophosphorylation. Is there a reason that this phosphorylation-based clock was built out of a hexameric ATPase? Why does KaiC have two catalytic domains, CI and CII? What is the functional role of CI in the circadian rhythm? How are input and output signals transduced into and out of the core oscillator without impairing rhythmicity?

Chang et al. present a structural and biochemical analysis of the physical interaction of the Kai proteins and the inner workings of the circadian clock. Using the more tractable thermophilic version of the system, the authors are able to separate the domains of KaiC and introduce mutations to control the oligomeric state of the proteins. This allows them to make two important discoveries regarding the previously structurally murky KaiB·KaiC interaction: first, that KaiB interacts with the CI ATPase domain of KaiC; and second, that the binding of KaiB involves some disruption of the ring structure of KaiC.

Previously the weight of evidence had suggested that KaiB made contact with the CII domain of KaiC. This ambiguity probably resulted from two sources: the structural similarity of CI and CII, and the requirement for CII phosphorylation, particularly on the key Ser431 residue, for KaiB interaction (11). The implication of a structure where KaiB instead interacts with the CI domain is that that the relationship between CII phosphorylation and KaiB binding cannot be as simple as the direct recognition of a phosphorylated peptide. Instead, the emerging picture is that information about the phosphorylation status of CII is transmitted through KaiC to CI via allosteric ring–ring stacking interactions (17).

The authors find that KaiB cannot interact with a tightly assembled CI ring and, in the case of the truncated CI protein, KaiB stably interacts with free CI monomers that have dissociated from hexamers. In full-length KaiC, ATP binding in the CI domain is the principal force that assembles the protein into active multimeric particles (9). This information suggests that destabilization of KaiC's hexameric structure is required for KaiB binding, helping to explain why exchange of monomers between KaiC particles occurs at the same time in the circadian cycle when KaiB interaction is strongest (12, 18).

In summary, the work of Chang et al. promotes an understanding of the structure of KaiC, where distinct functions are divided between the CI and CII domains. In this model, the daily oscillation of the system reflects an oscillation of enzymatic activity and protein–protein interaction between CI and CII. During the subjective

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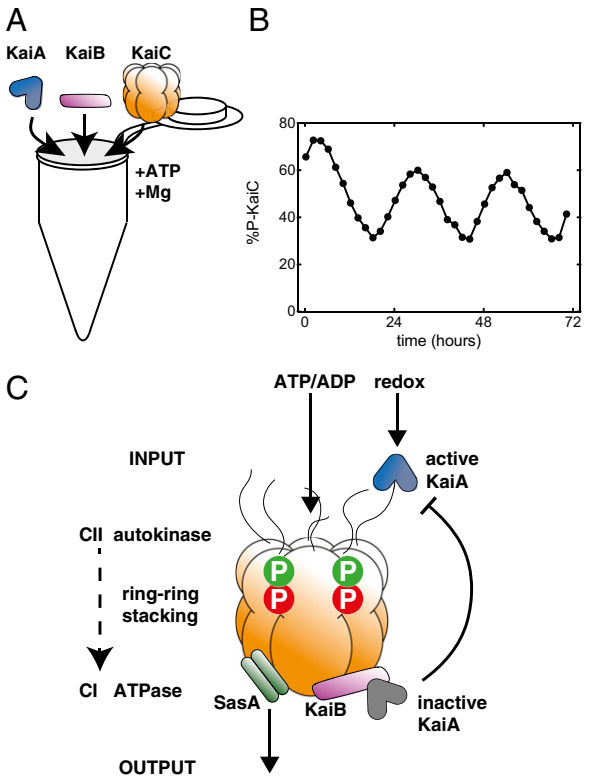

Fig. 1. Roles of two KaiC domains in the cyanobacterial clock. (*A*) Circadian rhythms can be reconstituted in vitro using a mixture of purified KaiA, KaiB, and KaiC in the presence of ATP. (*B*) Example in vitro rhythm in KaiC phosphorylation, reaction incubated at 30 °C. (*C*) Known biochemical interactions mediating input, output, and oscillations within the KaiABC system. Active KaiA binds to C-terminal tails of KaiC, switching CII from a phosphatase mode to a kinase mode. This process can be modulated by the ATP/ADP ratio or redox-dependent quinone interactions with KaiA. Multisite phosphorylation of CII on Ser431 and Thr432 induces ring–ring stacking interactions with CI, permitting KaiB to bind to CI. KaiA is sequestered in these KaiB complexes on CI, constituting a negative feedback loop on KaiC phosphorylation. The histidine kinase SasA also interacts with the CI domain, transducing transcriptional output from the circadian clock.

day, KaiA acts on the CII ring in its role as an activator of phosphorylation, whereas during the subjective night, KaiA relocates to inhibitory KaiA·KaiB·KaiC complexes on CI, permitting the clock to dephosphorylate.

This picture also suggests a rough division of KaiC into rings responsible for input and output functions. The best-understood input mechanisms involve modulation of CII activity by the ATP/ADP ratio and redox signals that act on KaiA, the activator of CII phosphorylation (19, 20). In contrast, the output histidine kinase SasA interacts with the CI domain. In this vein, the sequestration of KaiA in complex with KaiB on CI can be viewed as clock output signaling feeding back on clock input to produce free-running rhythms (Fig. 1*C*).

These data raise many important questions whose answers will likely be critical to understanding the principles underlying the biochemical origin of circadian rhythms. Because allosteric coupling between CII phosphorylation and CI is now clearly seen as a determinant of KaiB binding, what is the precise nature of the interaction between the two domains? Because disruption of the CI ring is important for KaiB binding, and the CI ring is held together by ATP, what is the functional role of the CI ATPase activity in the clock, already known to be temperature insensitive and linked to clock period? Along these lines, why does the assembly of KaiB·KaiC complexes take hours, even for KaiB kept in a binding-competent dimeric state? The slow kinetics of this process are likely fundamental to circadian timing, and our improved understanding of the nature of the KaiB·KaiC complex will shed light on this biophysical problem.

There are many ways to make a chemical oscillator, as is clear from non-biological examples, such as the Belousov-Zhabotinsky reaction (2), and examples from synthetic biology, such as the repressilator (21). Circadian clocks are a special class of oscillator that have presumably been shaped by natural selection to perform reliably and remain synchronized with the external environment under a wide variety of conditions. The existence of the purified KaiABC system, which shares many of the properties of circadian clocks in intact organisms, gives us the opportunity to see clearly the structure of the reaction networks that living organisms use to measure time.

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